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ORIGINAL ARTICLE

Phylogenetic and molecular analysis based on genes 16S-rRNA, OMPA and POMP to identify *Chlamydia abortus* infection occurrence at the milk samples of goats and sheep in west Azerbaijan of Iran

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ABSTRACT

Background and Objectives: Enzootic abortion in sheep and goats, also called ovine enzootic abortion (OEA) or enzootic abortion of ewes (EAE), is caused by *Chlamydia abortus*. The disease has a major economic impact as it represents the most important cause of lamb loss in sheep in parts of Europe, North America and Africa. This serious and potentially life-threatening zoonosis can also affect pregnant women after contact with lambing ewes, leading to severe febrile illness in pregnancy and loss of the foetus.

Materials and Methods: The present study was conducted to the Phylogenetic and Molecular Analysis based on Genes 16S-rRNA, OmpA and POMP of *C. abortus* in milk samples collected from sheep and goats in West Azerbaijan province, Iran. During 2018, a total number of 360 milk samples were collected from sheep (n = 180) and goats (n = 180) of different regions of the province. All milk samples were subjected to DNA extraction and examined by PCR.

Results: Among 360 milk samples collected from sheep and goats, 31 (8.611%; 95% CI=6.13-11.96) were positive for *Chlamydia* spp. The helicase, 16S-rRNA and ompA genes were examined and resulted in 8, 31, 31 of positive samples respectively. The accession numbers have been deposited in GenBank (NCBI) (MT367602 and MT367603).

Conclusion: Phylogenetic analysis based on the gene of helicase showed that most of the isolates shared similarity > 99.97%.

Keywords: *Chlamydia*; Nested polymerase chain reaction; Helicase; Outer membrane protein A (OmpA); Polymorphic outer membrane protein (POMP)

INTRODUCTION

Chlamydiae are intracellular micro-organisms that cause different diseases in man and animals. According to a classification based on the phylogenic analysis of their 16S and 23S rRNA genes, the family *Chlamydiaceae* consists of two genus, *Chlamydia* and *Chlamydophila* (1). Enzootic abortion of sheep, caused by *Chlamydophila abortus (C. abortus)*, is an infectious disease characterized by placentitis and abortion and has negative effect on sheep breeding in many countries. *C. abortus* infection is also observed in goats and cattle (2, 3). No clinical signs are present in the animals until abortion or delivery; animals who do not abort deliver very weak lambs (4). The abortion generally occurs in the last 2-3 weeks of pregnancy. It has been reported that the abortion

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rate in affected herds is low in the first year, reaches or exceeds 30% in the second and is of the order of 5-10% in the third year (1). Latent infections continuing longer than 3 years have also been reported (5). Direct microscopic examination, pathogen isolation, serological tests (complement fixation test (CFT), enzyme-linked immunosorbent assay (ELISA) and immunofluorescence), immunohistochemistry and DNA based methods (PCR and DNA microarray) are used for diagnosis (2). Serological testing is more frequently used in routine diagnosis because pathogen isolation is difficult and time-consuming. Cross-reactions between *C. abortus* and Gram-negative bacteria such as *C. pecorum* and *Acinetobacter* can be observed with CFT, which is recommended by the Office of International Epizooticc (OIE) (2). Recently, conventional and real-time PCR have been widely used to identify *C. abortus* in clinical samples. PCR methods for this are based on amplification of the chlamydial outer membrane protein genes ompA, omp1 and omp2, the polymorphic membrane gene pmp, genes encoding 16S rRNA and helicase, and the 16S-23S rRNA intergenic interval (6-9). Use of rapid and reliable diagnostic tests is important in rapid controlling of the disease. The extent of *C. abortus* infections in Urmia has generally been documented serologically, while studies on pathogen isolation are relatively rare. This study was performed to determine the extent of *C. abortus* infection in aborting ewes and goats in west Azerbaijan, and to compare pathogen culture and PCR methods for diagnosis.

MATERIALS AND METHODS

Milk sampling. A number of 360, sheep (n=180) and goats (n=180), milk samples were randomly collected from the regions of West Azerbaijan province during 2018-2019. A number of 160 milk samples were taken from flocks with abortion history and the other 200 samples lacked history of abortion. Samples were placed in ice pack and transferred to the microbiology laboratory of the Faculty of Veterinary Medicine, Urmia University.

DNA extraction. Milk samples were processed for DNA extraction according to the method described by White et al. (10). After transferring of samples to the laboratory, milk samples were centrifuged at 14000 rpm for 10 min in 50 mL falcon tube and removed the cream layer. The supernatant was discarded and the sediment was re-suspended using 50 mL sterile phosphate-buffered saline (PBS) (11). Samples were then centrifuged at 14000 rpm for 10 min. The supernatant was discarded and the precipitate was dissolved in 20 mL PBS solution and centrifuged for 10 min at 14000 rpm. The final precipitate was dissolved in 1 mL PBS and stored at -20°C until used for DNA extraction. To extract DNA, 200 µL of the final sediment solution was used. Genomic DNA was extracted using the Blood Genomic DNA Extraction Mini Kit (50 preps), (Favorgen, Taiwan), according to the manufacturer instructions. Genomic DNA extracted from each isolate was quantified using the NanoDrop 2000c (Thermo Scientific, USA) spectrophotometer and stored at -20°C for the next genomic evaluation.

Molecular detection of 16S rRNA, POMP 90-91B and OmpA genes. For molecular detection of *Chlamydia abortus*, Nested-PCR targeting the 16S rRNA gene was employed using the described by Daibata et al. (12) and Longbottom et al. (13, 14). Two pairs of CpaX-1-CpaX-2 primers targeting POMP gene were used for ampification (the POMP 90-91B bp 912 gene from 1541 to 2452-bp). The method of Borrel et al. was used to detect *C. abortus* in samples (15). Nested polymerase chain reaction (Nested-PCR) was used to examine the presence of *C. abortus* genomic DNA in samples. The sequence of primers used to amplify the POMP 90-91B gene, which encodes the outer membrane protein of *C. abortus*, was selected based on a study by Szymanska-Czerwinska et al. and Arif et al. (16, 17). For the molecular detection of *C. abortus* PCR targeting the *ompA* was employed. The primers for the PCR which were used in this study previously described by Creelan et al. (Table 1).

Normal PCR and nested-PCR for the detection of *Chlamydia abortus*. To perform the the Normal PCR and Nested-PCR, 16S rRNA, POMP and OmpA genes Taq DNA Polymerase Master Mix RED (Amplicon, Denmark) was used.

PCR detection of 16S rRNA gene. The PCR reaction was carried out in 25 µl volume comprising 5 µl of extracted DNA, 50 pmol of each primer (16SIGF, 16SIGR), 12.5 µl of master mix and 6.5 µl of distilled water. For the Nested-PCR stage, PCR reaction was prepared as the 16SIG-PCR which described previously except for the DNA template, which 2.5 µl of

1:100 diluted PCR product from the first stage was used. The thermal cycling condition was described according to Daibata et al. (12).

Amplification of Polymorphic outer-membrane protein (POMP) 90-91B gene by PCR. For PCR, the optimal concentration of materials used in the reaction in the final volume of 25 microliters is used as follows. 2.5 µl buffer, 2 µl template DNA (from previously DNA extracted) (4 ng DNA template per reaction), 1.5 mM $MgCl_2$, 1 µmol per primer (CpaX-1-CpaX-2 primers), (to dilute primers, 10 µl of each primer was mixed with 90 µl of sterile distilled water, 0.3 units of Taq DNA polymerase, and 1 µl of a mixture of dNTPs. All material was transferred to a microtube and placed in a thermocycler. Negative control involves a mixture of all PCR reagents without the presence of DNA and instead of DNA, sterile distilled water is added to the tubes. Positive control contained *C. abortus* standard strain (S26/3) DNA. Standard strain was supplied by the Pendik Veterinary Control and Research Institute (Istanbul).

Detection of OmpA gene in *C. abortus*. In each reaction, the final concentration of reagents was as follows; PCR reaction was carried out in 25 µl volume comprising 5 µl of extracted DNA, 50 pmol of each primer (ompA 1, ompA 2), 12.5 µl of master mix. Amplification conditions were described according to DeGraves FJ et al. and Creelman et al. (18, 19). All samples containing a band at 479-bp were Southern-blotted using standard techniques and the specificity confirmed using a strain-specific probe derived from the 479-bp fragment amplified using clone 8 strain-specific primers. This probe was peroxidase-labeled using the ECL direct nucleic acid-labeling system as described previously (20).

Detection of PCR products. PCR products of 16S rRNA, POMP 90-91B and OmpA genes were visualized on agarose gel of 1.5, 2 and 1% using gel (Syngene Bio-Imaging, UK) documentation, respectively.

Statistical analysis. The obtained data were statistically analyzed by Chi-square test using SPSS software Ver. 22 (SPSS Inc., Chicago, IL). The P value< 0.05 was considered significant.

Sequencing of DNA. The PCR product of helicase partial gene was sequenced by Macrogene Company

Table 1. Primers used in Normal-PCR and Nested-PCR to detect 16S-rRNA, POMP and ompA gene.

Target	Primer	Sequence 5/----3/	Gene Detected	Amplimer Length (bp)	PCR Conditions (Centigrade) / for Seconds or Minutes				PCR Cycles	References
					Pre Denaturation	Denaturation	Annealing	Extension		
16SIG-PCR	16SIGF	TACCTGGTAGGCTCAATT	16S-rRNA	436	95 C / 3 Min	94 C / 30 Sec	70 C / 30 Sec	72 C / 45 Sec	45	(12)
	16SIGR	ATAATGACTTCGGTTGTTATT								
16SIG nested-PCR	F	TGTTTTAGATGGCTAAACAT	16S-rRNA	127	95 C / 2 Min	94 C / 1 Min	55 C / 30 Sec	72 C / 1 Min	35	
	R	ACGGTCACTTGGAAACAAGG								
POMP 90-91B PCR	CpaX-1	AGCAGAGGTTGGGCTCACTA	POMP 90-91B	912	94 C / 3 Min	94 C / 45 Sec	56 C / 45 Sec	72 C / 45 Sec	30	(15)
	CpaX-2	TGGTATTCTTGCCGATGAC								
ompA	OMPA-1	GATCGTAACTGCTTAATAAACCG	OMPA	479	95 C / 3 Min	94 C / 30 Sec	70 C / 30 Sec	72 C / 45 Sec	45	(18)
	OMPA-2									

• C=Centigrade, Min=Minute, Sec=Second

of Korea using ABI Prism Terminator Sequencing Kit (Applied Bio system) at. Chromatograms of helicase were edited and base calls checked using Finch TV program software.

Sequence alignment and submission. The helicase gene sequences were aligned using Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Partial gene sequence of helicase genes were submitted to the Genebank under accession numbers MT367602 and MT367603.

RESULTS

Amplification of 16S rRNA, POMP and OmpA genes. Among 360 milk samples collected from sheep and goats, 31 (8.611%, 95% CI = 6.13-11.96) were positive for *Chlamydia* spp. amplifying a fragment of 127bp of the 16S rRNA gene using Nested-PCR (Figs. 1 and 2). The prevalence of *Chlamydia* spp. in the milk of two examined species was statistically significant. The prevalence of *C. abortus* infection was significantly different in terms of regions with the highest frequency in central region. Animals with abortion history showed higher number of positive milk samples for *C. abortus.* Overally, 31 samples (out of 360) were infected by either one of the pathogen. Among 31 positive samples (16S-rRNA gene), all samples were positive for *C. abortus* amplifying a fragment of 479 bp of the OmpA gene using PCR (Fig. 3). In this study, only 31 cases (8.611%, 95% CI = 6.13-11.96) were identified as *C. abortus* in sheep and goat by producing a 479 bp fragment using PCR. The helicase gene was examined subsequently. Among 31 positive samples (16S-rRNA gene), only 8 samples (25.81%, 95% CI = 13.7-43.25) (they were positive for *C. abortus* amplifying a fragment of 343 bp of the helicase gene using PCR. (Fig. 4). Phylogenetic tree constructed based on neighbor-joining analysis of helicase partial gene revealed that six isolates were closely clustered together showing 99.97% similarity which can be considered identical (Fig. 5).

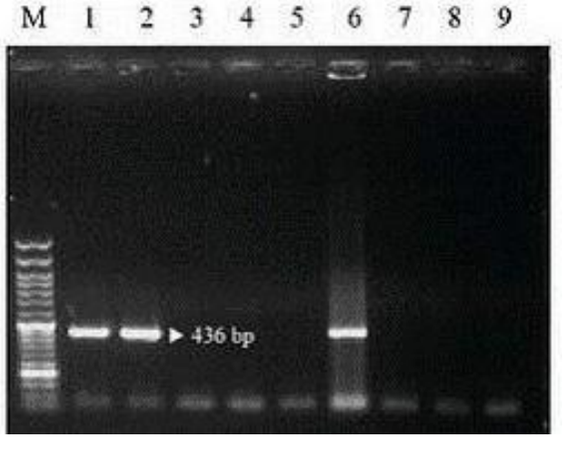

Fig. 1. Agarose gel image of amplified fragment of *C. abortus* 16S rRNA gene (463 bp) using Nested-PCR. Lane M, 50 bp molecular ladder (Smobio Technology Inc., Taiwan); Lane 1, Positive control (*C. abortus* S26/3 DNA); lanes 2, 6, positive samples for *C. abortus*; Lane 3, 4, 5, 7, 8 negative samples for *C. abortus*; lane 9, negative control.

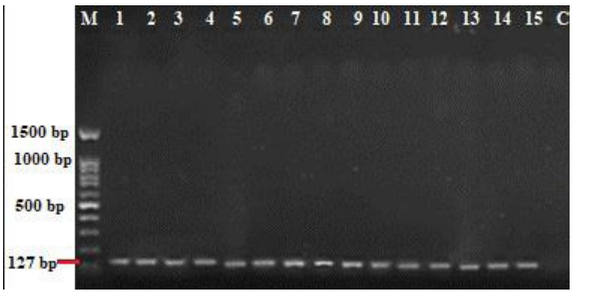

Fig. 2. Agarose gel image of an amplified fragment of the *C. abortus* 16S rRNA gene (127 bp) using Nested-PCR. Lane M, 100 bp molecular ladder (Smobio Technology Inc., Taiwan); Lane 1, Positive control (*C. abortus* S26/3 DNA); Lanes 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, positive samples for *C. abortus*; Lane C, negative control (distilled water).

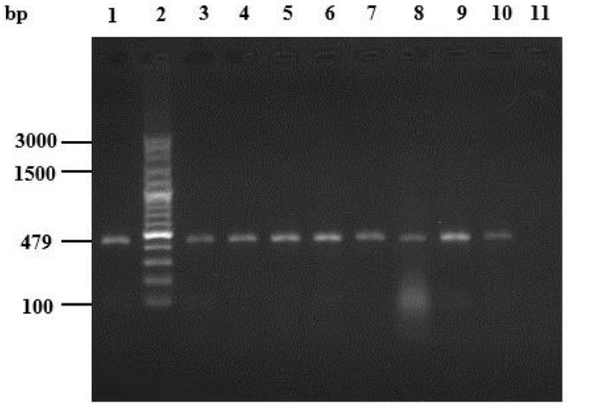

Fig. 3. Agarose gel form of gene fragment (*C. abortus* OmpA (479 bp) using PCR. lane 1, positive control (*C. abortus* S26/3 DNA); lane 2, 100 bp molecular leader (Smobio Tecnology INC., Taiwan); lines 3, 4, 5, 6, 7, 8, 9, 10 Positive samples for *C. abortus*; lane 11, negative control.

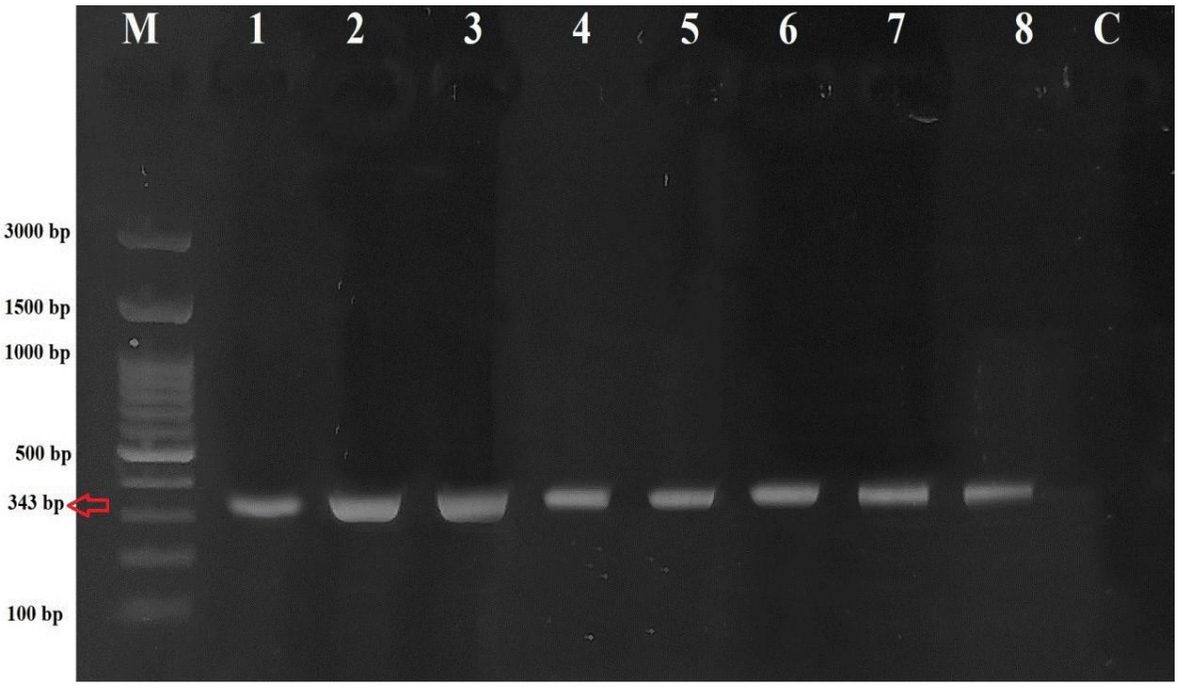

Fig. 4. Agarose gel image of amplified fragment of *C. abortus* helicase gene (343 bp) using PCR. Lane M, 100 bp molecular ladder (Smobio Technology Inc., Taiwan); Lane 1, Positive control; lanes 2, 3, 4, 5, 6, 7, 8, positive samples for *C. abortus*; Lane C, negative control samples for *C. abortus.*

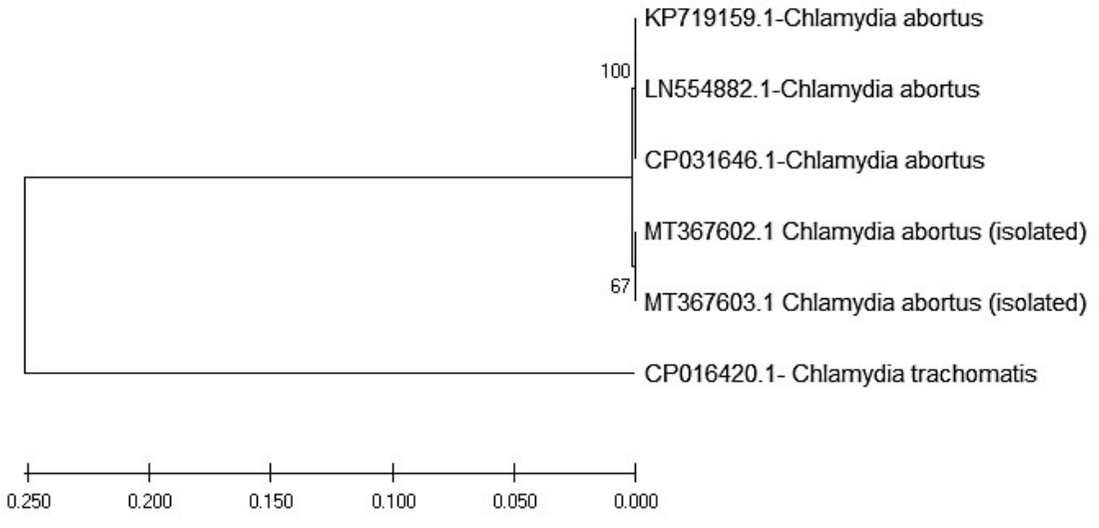

Fig. 5. Evolutionary analyses were conducted in MEGA X nblast show phylogenetic positioning of MT367602.1 and MT367603 *C. abortus* based on partial helicase gene that employing maximum likelihood available in GenBank sequences. Numders on nodes indicate the bootstrap values.

Molecular identification of *C. abortus*. The partial helicase sequence sample with size 343-bp are alimented by BLAST program (21) from Gen bank (http://blast.ncbi.nlm.nih.gov/) was used to compare our amplified sequence with other stored species of bacterial sequences. The results got from the BLAST indicated that the highest query sequence was 99.97% identical to *C. abortus* (Table 2).

Phylogenetic inferences. Phylogenetic analy-

sis based on helicase nucleotide sequence revealed grouping of investigated species of bacterial on expected lines. From sequence divergence similarity data and phylogeny constructed, it was revealed that species belonging to respective genera were close to each other. The sample specie grouped in clusters of *C. abortus* Genbank (Table 3). The relatedness bacterial sample with other Genbank bacteria species identified of Genbank were retrieved from MegaX software program (Fig. 5).

DISCUSSION

We have successfully developed a Nested PCR that can detect and differentiate *C. abortus*, with a good sensitivity and specificity. The diagnosis of chlamydiosis may be greatly simplified and performed at low cost. In addition, the improvement in diagnostic techniques will enhance our knowledge regarding the prevalence and the pathogenic significance of chlamydiosis. However, attempts to isolate *C. abortus* from milk of affected sheep have resulted in failure and the question whether the causative agent of OEA is excreted in milk is yet to be answered (22-25).

The prevalence of *C. abortus* in sheep and goats with an abortion history was 14.38% which was significantly higher than of those without abortion history (4%). This finding is consistent with the other studies reported from Iran and other countries (1, 4, 26). The reason for this finding is that protective immunity does not develop when non-pregnant sheep are infected, and it can result in abortion (24). Nucleic-acid based methodologies, in particular polymerase chain reaction (PCR) compare favorably with cell culture in terms of speed, easiness and safety. However, this technique may suffer from low sensitivity when dealing with clinical specimens including milk because of unknown inhibitors and difficulty in sample preparation. A variety of sample indentification methods including the Nested-PCR have successfully been applied to overcome these drawbacks and as a result to improve the sensitivity of the PCR assays (1, 4). In a recent study on the epidemiology of *Chlamydophila* infection in calves, Jee et al. (25). Detected *C. abortus* in the milk of 15% of dams without any signs of disease. One-hundred-microliter milk samples from a single udder quarter were tested per week for 12 weeks postpartum. Thus, the

Table 2. Tabular overview of BLASTN result including hits identifiers and scoring helicase sequences have detected *C. abortus* of bacterial samples.

Descriptions	**Graphic Summary**	**Alignments**	**Taxonomy**									
Sequences producing significant alignments								**Download**	**New Select Columns**		**Show**	**100**
Select all	**100 sequences selected**							**GenBank**	**Graphics**		**Distance tree of results**	
Description			**Common Name**	**Max Score**	**Total Score**	**Query Cover**	**E Value**	**Per Indent**	**Acc. Len**	**Accession**
Chlamydia abortus isolate FRI 2 helicase gene. partial.cds			*Chlamydia abortus*	619	619	100%	3e-173	100.00%	343	MT367603.1
Chlamydia abortus isolate TAH1 helicase gene. partial.cds *Chlamydia abortus* strain 84/2334 Chromosome. complete genome *Chlamydia abortus* strain GIMC 2006:CabB577, complete sequence *Chlamydia abortus* strain ChIa-IR-FM-104 putative helicase gene partial, cds *Chlamydia abortus* strain ChI- IR-FM-101 putative helicase gene, partial cds *Chlamydia abortus* strain GN6, complete genome			*Chlamydia abortus*	619	619	100%	3e-173	100.00%	343	MT377602.1
			Chlamydia abortus	615	615	100%	1e-171	99/71%	1165692	CP031646.1
			Chlamydia abortus	615	615	100%	1e-171	99/71%	1144224	CP024084.1
			Chlamydia abortus	615	615	100%	1e-171	99/71%	441	KP719159.1
			Chlamydia abortus	615	615	100%	1e-171	99/71%	441	KP719156.1
			Chlamydia abortus LLG	615	615	100%	1e-171	99/71%	1144357	CP021996.1
Chlamydia abortus LLG genome			*Chlamydia abortus*	615	615	100%	1e-171	99/71%	1143694	CP018296.1
Chlamydophila abortus strain 1H genome, assembly chromosome: 1			*Chlamydia abortus*	615	615	100%	1e-171	99/71%	1144467	KN554883.1
Chlamydophila abortus genome assembly CAAB7, chromosome: 1			*Chlamydia abortus* S26/3	615	615	100%	1e-171	99/71%	1144467	LN554882.1
Chlamydophila abortus strain S26/3. complete genome				615	615	100%	1e-171	99/71%	1144377	CR848038.1

Table 3. Percentage distribution of isolated bacterial with accession numbers MT367602.1 and MT367603 *C. abortus* of partial helicase gene according to nblast that available in Genbank of NCBI.

Submitted Isolated Samples	Query Cover %	Identic Number %	Genbank Accession Number	Genbank Bacterial Identification	Country Identification
	100	99.71	CP031646.1	*C. abortus*	Germany
MT367602.1	100	99.71	KP719159.1	*C. abortus*	Iran
MT367603.1	100	99.71	LN554882.1	*C. abortus*	United Kingdom

sampling intensity was low, and a higher prevalence of *Chlamydophila* spp. in milk might be detected with a higher sampling intensity. Nevertheless, these results indicate that low-level natural infection of the bovine mammary gland with *Chlamydophila* spp. most likely is common. The previous studies showed that the seroprevalence of *Chlamydia* spp. infections in the asymptomatic ovine population was 1.15%, while in cases with ovine reproductive disorders it was 1.20%. Results of the present study revealed that 8.11% of all examined raw milk samples were positive for *Chlamydia* spp. The findings of the current study are in agreement with the similar studies from Iran and the other countries. Serological studies showed that 25% of sheep flocks of Share-Kord provinces in Iran had antibodies against *C. abortus* respectively (26). Pinheiro Junior et al. showed that 21.5% of sheep in Alagoas-Brasilia had antibodies against *C. abortus* and 77.7% of the population had at least one seropositive animal (26). The Nested PCR study confirmed the presence of *C. abortus* in the tested samples. The presented study indicates that infections with *Chlamydia* spp. are present among sheep and goats in Iran, but the percentage of infected animals is not high.

CONCLUSION

The prevalence of *C. abortus* infection in sheep and goats milk was determined for the first time in West Azerbaijan, Iran. The results of the Phylogenetic analysis showed that there is no significant difference between isolates in Iran and isolates in different parts of the world, including Germany and the United Kingdom. The molecular detection of *C. abortus* using the Nested-PCR method in milk samples showed that PCR can be used as an easy and reliable approach for detecting *C. abortus.* The prevalence of *C. abortus* was high in sheep and goats milk. Therefore, the consumption of sheep and goats milk infected with *C. abortus* exposes humans to a higher risk of *Chlamydial* infection.

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